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Author: H. Jockusch, Max Planck Institute for Biology, Melchers Section (Max-Planck-Institut fur Biologie, Abt. Melchers), Correnssstrasse 41, Tubingen, Germany.

Title: Temperature sensitive mutants of the tobacco mosaic virus. I. In vivo behavior (Temperatursensitive Mutanten des Tabakmosaikvirus. I. In vivo-Verhalten).

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October 1968

A. INTRODUCTION

I. Structures, which are composed of helically arranged globular protein subunits, are widely distributed among animals, plants, bacteria, and viruses. Their analyses and reconstitution outside the cell is in many cases successful through appropriate alterations of the physicochemical environment (for examples, see Szent-Gyorgyi, 1951; Audley and Cockbain, 1966; Abram and Koffler, 1963; Poglazov, Borhsenius, and Belavtseva, 1965). In the case of the tobacco mosaic virus, the entire virus particle constitutes such a structure whose accurate or approximate structure can be deduced from studies on alkali-digested virus (Schramm, 1947), nucleic acid-free sheath protein alone (Takahashi and Ishii, 1952), or sheath protein together with intact RNA (Fraenkel-Conrat and Williams, 1955). The disaggregation and reaggregation studies have shown that the protein ultrastructures are not held together by covalent bonds and that the properties of the subunits determine their geometric arrangement. The capacity of the subunits to form ordered aggregates can be destroyed like the catalytic activity of enzymes by forces which do not involve covalent binding (denaturation - for definition, see Joly, 1965). In the case of the tobacco mosaic virus, through studies on the renaturation of phenol- and urea-denaturated sheath proteins, it has been concluded that the amino acid sequence alone probably determines the folding of the subunits and consequently the morphological structure of the virus particles (Anderer, 1959). The sheath protein of TMV is both structurally and protein-chemically the best studied structural protein (for structural

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Abbreviations used: TMV = tobacco mosaic virus; ts = temperature-sensitive; tr = temperature resistant; def = defect; SP = sheath protein; RNA = ribonucleic acid; RNase = ribonuclease; EDTA = ethylenediamintetraacetic acid.

studies, see Anderer, 1963, and physicochemical studies, see Caspar, 1963). Through studies on the genetic code, mutants with one or several known amino acid substitutions are available (Wittmann, 1962, 1964; Tsugita and Frankel-Conrat, 1962). Through physiological studies (Jockusch, 1966a), it has been shown that many of Wittmann's nitrite-mutants and several spontaneous mutants of TMV (Melchers, 1942 and unpublished) are temperature-sensitive (ts): in comparison to the wild types and temperature-resistant (tr) mutants, they produce considerably less extractable, infectious virus particles at 30-32°C than at 23°C (Jockusch, 1964). Kassanis (1957) has made similar observations in the case of tomato mosaic virus, which is a natural variant of TMV. It was possible for him to show that high temperatures did not have any effect on either the primary events of infection or the finished virus particles; he was not able, however, to present an explanation for this temperature susceptibility.

II. The appearance of temperature sensitivity through a single mutation was first shown in Neurospora (Mitchell and Houlahan, 1946). An experimental explanation for the mechanism was presented by Maas and Davis (1952) for a temperature-sensitive, auxotrophic mutant of Escherichia coli. In this case, the pantothenate synthetase of the mutant, which required pantothenic acid at high temperature, was essentially more thermal-labile in crude extracts than was the enzyme from the wild type. In the case of structural proteins, mutations leading to temperature sensitivity were first demonstrated by Edgar and co-workers in the T_4 bacteriophage system using genetic methods (Epstein et al., 1963; Edgar and Lielausis, 1964). Using the Polio virus, Cooper (1962) demonstrated a correlation between the temperature optimum for intracellular virus replication and urea-resistance of the virus particles. Since these strains have been obtained by selection at different temperatures, then a compatible alteration

of the stability of several virus-specific products cannot be excluded[1]. A correlation of *in vivo* and *in vitro* behavior, similar to the findings of Maas and Davis, was first clearly shown for a structural protein using the sheath protein of TMV mutants. This has already been reported on (Jockusch, 1964, 1966b).

III. By means of the studies described in this and the subsequent paper, it has been shown that the *ts* behavior of the predominant majority (class I) of the *ts* mutants of TMV (only) is based upon amino acid substitutions in the sheath proteins, and that the *in vivo* behavior can be explained by experimental studies *in vitro*, and that the influence of some external conditions and known amino acid substitutions on the thermal stability of TMV sheath protein can be demonstrated.

B. MATERIAL AND METHODS

I. Source of Biological Materials

1. The host plants employed and the virus strains are presented in Tables 1 and 2 along with information as to the source.

2. Isolation of New Temperature Mutants

Nitrous acid was employed as the mutagen (Mundry and Gierer, 1958). Directions for its employment are given by Wittmann (1962). Strain Al4 served as the parent strain. In the case of the Series 22.... and Ni23....., lesions,

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[1] A specific correlation between *in vivo* temperature sensitivity and lability of virus particles was demonstrated nevetheless in the case of the Sindbis virus (Burge and Pfefferkorn, Virology, 30, 1966).

TABLE 1

Host Plants

Name	Family	Source
Nicotiana tabacum var. Samsun (systemic host)	Solanaceae	see Melchers et al., 1966
Nicotiana tabacum var. Xanthi (local lesion host)	Solanaceae	Takahashi, 1956.
Plantago major	Plantaginaceae	grown in Tubingen
Lunaria annua	Cruciferae	purchase in Tubingen
Amaranthus paniculatus (var. "Dwarf torch"	Amaranthaceae	purchased in Tubingen

TABLE 2

Virus Strains

Designation	Parent Strain	Mutagen	Isolation
Vulgare - Nic. virus I*	--	--	Johnson, 1926
Ni103 - 120	vulgare	HNO_2	Wittmann, 1962
Ni1688 - 2068**	vulgare	HNO_2	Wittmann, 1964
FU 27	vulgare	F-uracil	Wittmann, 1964
CP 415	vulgare	--	V. Sengbusch, 1965
PM 2	vulgare	HNO_2	Siegel et al. 1962
flavum	vulgare	--	Melchers, 1942
necans	flavum	--	Mundry, 1957
reflavescens	necans	--	Melchers, unpubl.
A-14	vulgare	--	Melchers, unpubl.
Ni458 - 2196	A 14	HNO_2	Wittmann, 1962
Ni2204 - 2519	A 14	HNO_2	Jockusch & Wittmann, unpublished

Table continued on next page

Table 2, Contd.

Designation	Parent Strain	Mutagen	Isolation
dahlemense	--	--	Melchers, 1940, 1942
U 2 (mild)	--	--	Wildman et al. 1951
Holmes' Rib grass (HRG)	--	--	Holmes, 1941

* Found in 1937 by Stanley

** In Jockusch, 1964 and Wittmann-Liebold et al. 1965, mistakenly described as derived from A 14.

Text, continued from Page 3

which were produced on Xanthi tobacco after nitrous acid treatment, were each inoculated onto Samsun leaves. Parallel halves of these leaves were incubated in a climate chamber at 23°C and 32°C in nutrient solutions. The virus concentrations produced on the leaf halves were then determined in a comparative manner on Xanthi tobacco by the local lesion method. By means of this test, mutants were selected which showed significant deviations from the parent strain A 14. They were further tested as described in Section C.I.

In the case of series Ni25..., these were specially selected from mutants, which themselves did not display tissue infectivity at high temperatures. Like the test employed by Edgar and Lielausis (1968) for T_4 coli phage and considering the fact that the hypersensitive tested used as an indicator on Xanthi tobacco is in itself temperature-sensitive, the mutant mixture was commonly first inoculated onto the xanthi tobacco at high dilutions. The plants were then held for one day at 23°C, followed by 2 days at 32°C, and then two additional days at 23°C for "development" of the necrosis. Those lesions were

selected whose edges were not clearly dispersed as is the case with tr and ts-I strains grown at 32°C ("inflammed spot necrosis"). In contrast to the plaque technique for phage, during this process, a selection was made against those mutants whose temperature sensitivity involves the maturation of the virus particles, since complete virus particles are not necessary for the diffusion from cell to cell in the leaf tissue (Siegel, Zaitlin, and Sehgal, 1962). The isolation of the mutants was carried out jointly with Dr. H.G. Wittmann (Max Planck Institute for Molecular-Genetics).

3. Recovery and Purification of Virus

The method employed here is described in the papers by Wittmann (1962 and 1964). For the removal of plant proteins, a Frigen 113 (Hoechst)[2] was employed. A few preparations were recovered by the polyethylene glycol method (Leberman, in press). In all cases, with exception of the recovery of the mutant Ni2204, anheat precipitation of the plant proteins for 10 minutes at 60°C was carried out. This is no indication that this method of removal of the plant proteins has any effect on the protein-chemical properties of the purified virus preparations (see V. Sengbusch, 1965).

II. Sera

Antiserum against TMV-vulgare was prepared using Freund's adjuvant as described by V. Sengbusch (1965). For titering, diluted samples were tested against the antigen and the formation of a visable precipitate checked for.

III. Climate Chamber

The climate chamber employed was described by Mundry (1957). The temperatures given were air temperatures whose possible variation was about ± 0.5°C.

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[2] The firm Farbserke Hoechst is thanked for the donated Frigen (translator's note: the word "Frigen" appears to be some sort of a trade name. It cannot be translated into English).

Under the test conditions employed, the deviation of the true leaf temperature from the air temperature should be < 0.5°C (Mundry, 1957). The chamber was continually illuminated with light of about 5,000 Lux intensity (Osram fluorescent lamps HNG 200)[3].

IV. Test Methods

1. Test for Stable Infectivity

Where not otherwise noted, this was carried out as follows: from each of the infected leaves to be tested were stamped out five discs (diameter 13 mm, weight ca. 30 mg each) which were then homogenized in a mortar with 0.25 ml of M/15 phosphate buffer, pH 7.0. 0.5 ml of the homogenates were diluted with 4.5 ml of phosphate buffer, allowed to stand for approximately one hour at room temperature, and then frozen at -10°C. 1 ml of test solution contains about 6 mg of leaf material (wet weight). For the test, the samples were thawed and the denatured plant protein was centrifuged out. The supernatant was applied to Xanthi tobacco with a glass rod using the half leaf method (for example, 32°C sample against a 23°C sample). The test plants were incubated in the green house.

2. Test for Virus Propagation in Tissues.

Generally, the primary symptoms on Samsun tobacco (chlorosis) were so clear at high temperatures that a special test for propagation of infectiousness was not required. In doubtful cases, an alternative test procedure on Xanthi tobacco was employed. The primary necrotic lesion on tobacco is in itself quite severe at high temperatures (Samuel, 1931). It can be "developed"

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[3] The Osram firm is thanked for the fluorescent lamps which were donated.

however, in about one day through reverse propagation at low temperatures. Accordingly, the TMV strain to be tested was inoculated onto the Xanthi plant, which, after 1 hour at room temperature, was held for the first two days at 32°C and then for one day at 20 -25°C. As the infectious agent diffuses through the tissue, large necrotic lesions (diameter 4-8 mm) are formed, whereas at lower temperatures, the controls did not give these typical necrotic lesions.

3. Electron Microscopic Examination for Virus Particles.

For this, leaf extracts prepared by low speed centrifugation were subjected to agar filtration (Kellenberger and Arber, 1959). The preparations were shadowed at an angle of 20° with platinum-palladium and examined under an Elmscope I (Siemans). (The electron microscopic examinations were carried out by Dr. H. Frank, Max Planck Institute for Virus Research).

4. Test for Labile RNA

The buffers of Sarkar (1965) were employed. In these the TMV-RNA in leaf extracts could be protected against RNase activity without the TMV virus particles being decomposed. Both RNase-sensitive and RNase-resistant infectious agents were differentiated in similar ways as described by Sarkar (1965). The buffers employed were: "NP" = 0.1 M phosphate, 0.5 M NaCl, pH 7.5, and "TP" = 0.01 Tris, 0.05 M Na_2HPO_4, HCl, pH 8.8.

5. Gel Electrophoresis of Proteins.

For the detection of TMV sheath protein polypeptide chains in plant extracts, electrophoretic separations were carried out in polyacrylamide gels using concentrated urea solutions (Duesberg and Rueckert, 1965), since in this case the original native state of the proteins does not play a role.

The apparatus developed by Tichy (1966) for the simultaneous electrophoresis of multiple samples was employed. The gel in Aronsson-Gronvall buffer (Aronsson-and Gronvall, 1957) contained: 10 % cyanogen 41 (Serva), 0.6 % diaminopropionitrile, 0.06 % ammonium peroxydisulfate. The polymerization was carried out at room temperature and took only a few minutes. During the run, the temperature was held at about 10°C by water cooling. The voltages was 200 V and the current was between 48 mA and 30 mA. The length of the run was 15 hours.

C. RESULTS

I. Temperature Behavior in Tobacco Tissues

The temperature behavior in tobacco of 26 mutants and four wild strains is given in Table 3, columns 3-7. The quotient Q denotes the ratio of the infectivity at 32°C to that at 23°C. Strains having a Q ratio of ≤ 0.5 are described as temperature-sensitive (ts). Strains which had a virus concentration at 23°C comparable to that of vulgare and which had a Q value of > 0.5, were classified as temperature-resistant (tr). Strains which produced far less virus particles than vulgare at 23°C were described as defective (def). The results for three independent studies carried out under different conditions are presented. The Q values are regarded as approximate values. The classification of several mutants as semi-sensitive based on the results of Study I (Jockusch, 1964) was discarded. The solutions tested in Study I were five times more concentrated (ca. 30 mg/ml leaf material, wet weight) than those used in the other studies (ca. 6 mg/ml leaf material, wet weight). Because of this, the Q values often turned out to be too high since the tests were not carried out in the linear region of the standard curve of number of lesions against virus concentration. By means of these Q values, however, (a) ts mutants can be specifically characterized, and (b) the relative stability of these ts mutants can be ascertained.

In the case of several mutants (for example Ni2068), the later studies show differences in the Q values which cannot be explained either as a result of non-linearity of the standard curve or as a statistical error (five leaf discs of 13 mm diameter containing >20 primary lesions). In these cases, the denaturation temperatures (see Kauzmann, 1954) probably lie in the vicinity of 32°C so that small variations in conditions during virus cultivation are substantially magnified.

The most important results in Table 3, columns 1-7, 13, and 14 are as follows:

(a) More than half (14 out of 21) of the mutants specified (the selected mutants of series 2,,.. are not included) are ts. In comparison, the four wild strains studied, namely, vulgare, dahlemense, HRG, and U2, are tr.

(b) With the exception of the special mutants Ni25...., which were selected against this characteristic, infectivity was displayed in all cases at 32°C on the primary infected leaf (distinct chlorosis on Samsun leaf or positive results for the tests described in Section B.IV.2.). These results show that the temperature-sensitive step can be neither a primary process of infection nor a step necessary for the intracellular synthesis of infectious materials.
Definition: ts mutants, whose infectiousness at high temperatures in tissues is displayed, should be hereafter described as ts mutants of class I (ts-I).

(c) Of the sixteen ts-I or def mutants, only three are not typical yellow strains: Ni 116, Ni 118, Ni 2068, However, Ni 116 and Ni 2068 still belong to relatively stable ts-I strains. Moreover, up until now, no strong yellow strain has been found which is not ts. On the other hand, between one other characteristic of TMV mutants, namely, to show necrosis on Java tobacco (Melchers, 1942; table for mutants used here from Wittmann, 1962, 1964) and the ts behavior, no significant correlation can be shown.

(d) All of the ts-I mutants have at least one amino acid substitution in their sheath protein as com ,red to their tr parent strains. (Protein-chemical data in this case are the papers by Wittmann, 1962, 1964; Wittmann-Liebold et al. 1965). This correlation, however, does not appear to hold true for mutants N i 511 and Ni 606 which have been described as "not having any amino acid substitution as compared to A 1h".(Wittmann, 1962). According to electrophoretic investigations on intact virus particles, these mutants have a substitution of the type: amino dicarboxylic acid → amino dicarboxylic acid amide, which cannot be detected after hydrolysis of the proteins (V. Sengbusch, 1965).

II. Variation of The Host Plant

The host range of TMV is extended over many plant families (Holmes, 1964). As a result, one can differentiate between the host-specific and the virus-specific characteristics by varying the host plant within as wide a range as possible. In a general study, the host plants Plantago major, Amaranthus paniculatus, and Lunaria annua were employed. Columns 8-13 of Table 3 show that generally when a TMV strain is ts on tobacco, then it is usually ts for these other three hosts; when it is tr, then it is tr on the other three hosts. The exceptions are: Ni 109 - tr on tobacco, ts on Amaranthus and Lunaria; reflavescens - ts on tobacco and Amaranthus, tr on Plantago; Ni 1196 - tr on on Plantago, ts on the three other hosts.

III. Proof for The Hypothesis, That The ts-Characteristic of The Class I Mutants is A defect affecting Maturation

1. Formation of Virus Rods.

For the different studies, electron microscopic checks were carried out as described in Section B.IV.3. It was found that vulgare produces considerably

more virus rods at 32°C than at 23°C after 4 to 6 days. The ratio of the rod concentration at 32°C to that at 23°C is significantly higher than the ratio of infectiousness at 32°C to that at 23°C. This indicates that the rods formed at the higher temperature have less specific infectiousness (see Lebeurier and Hirth, 1964). At 32°C, Ni 118 produces ≤ 1%, reflavescens ≤ 5 %, the quantity of rods formed at 23°C. At 30°, U 2 produces a quantity of rods comparable to that of vulgare, whereas Ni 118 produces < 1 % and flavum < 5 % the rod concentration produced by vulgare. These observations made on the three ts-I mutants and the two wild strains led to the conclusion that in the case of the ts mutants, the formation of rod-like sheath protein aggregates is temperature dependent.

2. Temperature Growth Studies.

(a) Pre-Incubation at High Temperature.

If the temperature defect were due to an indirect influence on the host cytoplasm, for example, the accumulation of metabolic products which inhibit the biosynthesis of certain TMV mutants, then a pre-incubation of host plants at high temperature should also have the same effect.

Seven week old Samsun plants were held at 23°C and 35°C in climate chambers (continous illumination). After the sixth day, there were inoculated with a 0.1 % Ni 118 virus suspension as in the test for temperature sensitivity, incubated for two days at 23°C, and then extracted and tested as described. The extracts of the plants held at the high temperature were compared with the controls in concentrations of 1/1, 1/5, and 1/25. The content of infective agent of the plants held at 35°C vs. that of the plants held at 23°C was Q = 0.77 ± 0.125 (twelve values from three different sample pairs). The heat treatment had then, at least in the case of Ni 118, no effect which would explain the temperature behavior of these strains on the basis of a persistent inhibitor material.

TABLE 3. Temperature-Behaviors of 4 wild strains and 26 TMV mutants

Strain, mutant	Amide Acid Substitution in Sheath Protein	Temperature Behavior on Nicotiana tabacum: Yellow Symptoms	QI	QII	QIII	Symptoms at 32°C	Plantago Q	Plantago Symptoms at 32°C	Amaranthus Q	Amaranthus Symptoms at 32°C	Lunaria Q	Lunaria Symptoms at 32°C	Correlation Nicotiana 23 and 32°C: Ts/amino-Ts/Yellow Acid Substitution		Classification: Type	Class
1	2a	3a	4	5	6	7	8	9	10	11	12	13	14	15	16	17
vulgare	/	0	1—2	1,0	1,5	+	1,2	0	0,5	+	1,0	0	+	+	tr	/
Ni 103	+	++	0,2	<0,01	–	+	–	–	<0,01	+	<0,01	+	+	+	ts	I
Ni 105	0	0	1,0	–	–	+	1,0	0	–	–	–	–	+	+	tr	/
Ni 106	0	0	1,4	0,6	–	+	1,0	0	–	–	–	–	+	+	tr	/
Ni 109	+	0	1,0	1,0	–	+	–	–	0,1	+	<0,01	0	+	+	tr	/
Ni 116	+	0	–	0,3	–	+	–	–	–	–	–	–	+	0	ts	I
Ni 118	+	0	<0,01	<0,01	–	+	0,06	+	<0,01	+	<0,01	+	+	0	ts	I
Ni 1688	+	+	0,5	0,05	–	+	–	–	–	–	–	–	+	+	ts	I
Ni 1927	+	0	1,5	1,0	–	+	–	–	–	–	–	–	(+)	+	tr	I
Ni 2068	+	0	1,0	0,02	0,03	+	–	–	–	–	–	–	+	0	ts	I
Fu 27	+	0	1,0	1,0	–	+	–	–	–	–	–	–	(+)	+	tr	I
OP 415	+	++	0,3	0,01	<0,05	+	–	–	<0,05	+	–	–	+	+	ts	I
flavum	+	++	<0,01	0,01	–	+	–	–	<0,01	+	<0,01	+	+	+	ts	I
necans	+	+	0,2	–	–	+	–	–	–	–	–	–	+	+	ts	I
reflav.	+	++	<0,01	<0,05	–	+	0,8	+	<0,01	+	–	–	+	+	ts	I
A 11	+/0	0	1,2	0,9	–	+	1,0	+	1,3	+	0,8	+	(+)	+	tr	/
Ni 458	+	+	0,2	0,01	–	+	–	–	<0,5	+	–	–	+	+	ts	I
Ni 511	+b	++	0,02	<0,01	–	+	–	–	0,2	+	<0,01	+	+	+	ts	I
Ni 606	+b	++	0,01	<0,01	–	+	0,01	+	–	–	–	–	+	+	ts	I
Ni 690	+	++	0,01	–	–	+	–	–	–	–	–	–	+	+	ts	I
Ni 1193	+	0	1,5	1,0	–	+	2,0	+	–	–	–	–	+	+	ts	I
Ni 1196	+	+	0,5	<0,01	–	+	1,0	+	0,03	+	0,05	+	(+)	+	tr	/
Ni 2204	+	++	–	<1 (def.)	–	+	–	–	def.	+	def.	+	+	+	ts	I
Ni 2239	+	+	–	<0,01	<1 (def.)	+	0,03	+	–	–	–	–	+	+	def.	I
Ni 2338	–	0	–	5,0	>10	+	>1 def.	(+)	0,05	+	1,0	+	/	/	ts/def	I
Ni 2516	0	0	–	0,2	–	?	–	–	<0,01	+	<0,01	0	0	0	ts	?
Ni 2519	0	0	–	0,15	–	0	–	–	<0,01	+	<0,01	0	0	0	ts	(III)
dahlem.	/	0	–	0,77	1,0	+	–	–	~1	+	–	–	/	+	tr	II
U 2	/	0	–	–	1,0	+	–	–	–	–	–	–	/	+	tr	/
H RO	/	0	0,7	–	0,73	+	–	–	–	–	–	–	/	+	tr	/

Legend to Table 3

The property over each column is given for the strain in question using the following symbols: + present; 0 absent; -- not studied; / not definite.

[a] The protein-chemical data in column 2 are data from the papers of Wittmann, 1962, 1964, and Wittmann-Liebold et al., 1965. Column 3: most of the descriptions of symptoms were from the papers of Wittmann, 1962, 1964.

[b] Substitution found by electrophoresis of the complete virus (V. Sengbusch, 1965.)

Column 1: For the genetic origin of the strain, see Table 2.

Column 2: Amino acid substitution in the sheath protein as compared with the parent strain.

Column 3: ++ strong yellow symptoms (example is flavum), + distinct yellow symptoms (example Ni 458), 0 no yellow symptoms. The distinct primary chloroses and the yellow area on sprouting, secondarily infected leaves are typical of the yellow strains. Ni 2338 shows primary necroses on Samsun tobacco.

Column 4-6: The quotients given are those obtained by the half leaf-test for the infectiousness of the 32°C sample/infectiousness of the 23°C sample. Study conditions: two leaves were cut from 7 week old Samsun plants which had been cultivated at 23°C for four days under continous illumination. They were rubbed with carborundum, inoculated with a 0.1 % virus suspension using a glass rod, and immediately washed with water. After 1 to 2 hours at room temperature, the plants were placed in the climate chamber. They were incubated in the case of Run I for four days and in the cases of Runs II and III for six days. In Run II, the plants were not placed in a climate chamber but were held in the green house at 20 - 25°C under continous illumination. Concentration of the extracts: In Run I ca. 30 mg/ml and in Runs II and III, ca. 6 mg fresh leaf material per ml buffer. For details, see text.

Column 7: + either symptoms clearly seen on Samsun tobacco at 32°C (as observed in most cases) or positive according to Test B.IV.2.

Column 8-13: Q values as previously defined. Inoculation as above. The symptoms that developed were always obtained from the same leaf which was also extracted. Plant ages: Plantago ca. 10 weeks, Amaranthus 3 weeks, Lunaria 6 weeks. Incubation times: Plantago and Amaranthus 4 days, Lunaria 7 days. Instead of five leaf cuttings as with tobacco, the entire leaf cutting (5-7 cm long) were extracted since significantly fewer primary foci were found in contrast to tobacco.

Column 14: +: tr and no amino acid substitution in the sheath protein or ts with a substitution; (+): tr with a substitution; 0: ~~remaining combinations~~ ts without substitution.

Legend to Table 3, Continued

Column 15: +: *ts* and yellow strain or *tr* and no yellow strain, 0: remaining combinations.

Column 16: *ts* $Q \leq 0.5$; *tr*: $Q > 0.5$ (in most studies); *its* $Q \gg 1$; *def*: also at low temperature significantly less infectivity produced than with *vulgare*. See text.

Column 17: See text.

(b) Post-Incubation at High Temperature.

If one permits the plant cells to synthesize virus at low temperatures and then transfers them to a higher temperature at a time when any additional increase in the number of viruses would be quite small, then a conclusion can be drawn on the basis of the virus titers obtained as to the intracellular stability of the mature virus particles. Included in this study also was Ni 2519 which does not belong to Class I.

Three leaves were cut from each of four 38 day old Samsun plants and were inoculated with the following virus suspensions (without carborundum): vulgare, Ni 118 - 0.1 % virus suspension in phosphate buffer, Ni 2519 - homogenized leaf material in phosphate buffer. The plants were kept in the green house for nine days (55 % of the time: temperature $\leq 25°C$, 45 % of the time: temperature $> 25°$ but $\leq 30°C$). Two plants were extracted as controls while the other pair was held for six days at 32°C in a climate chamber and then extracted. Extraction was carried out as described in Section B,IV.1., except that the samples were diluted 20-fold in the second step so that the final solutions would contain about 3 mg of fresh leaf material per ml. These solutions were further diluted 1/5 and 1/10 and tested in pairs (heat-treated vs. controls). The following contents of infectious agent were found:

Vulgare	$Q = 1.1 \pm 0.15$, $n = 2$[4]
Ni 118	$Q = 0.57 \pm 0.09$, $n = 4$
Ni 2519	$Q = 1.7 \pm 0.4$, $n = 3$

In contrast to that of vulgare, the Q value of Ni 118 is significantly lower but not so low that it explains the value of $Q < 0.01$ found by the test for temperature sensitivity (C.1.). The ts behavior according to this is not a consequence of the decomposition of mature virus particles in the cell. Under these conditions, a similar study with flavum could not be carried out since the leaves decay during the long incubation period. Kassanis (1957) has shown

[4] Mean $\pm$ standard deviation, n = number of parallel tests

however, that with the yellow strain, Tomato Acuba virus, which one must place in Class ts-I based on his information, the intracellular stability of mature virus particles at temperatures where no new virus particles can be produced, is maintained. The Q value of Ni 118, which is less than 1, can be explained from a comparison with the behavior of vulgare in two ways: (a) The virus particles of both strains are decomposed slowly at high temperature but vulgare compensates for this degradation by new synthesis. The findings of Kassanis (1957) support this possibility; (b) At temperatures over 25°C, the Ni 118 virus particles which are synthesized are more labile than those of vulgare. This possibility is improbable because of the extremely high in vitro stability of Ni 118 particles synthesized at fluctuating temperatures.(Part II, C.VI.). In all the cases, it was shown that eventual differences in the stability of mature virus particles cannot be the cause of this, and that Ni118 produces significantly less virus particles than vulgare at 32°C. The same holds true for Ni 2519. Since the maturation is not ts, prehaps this explains the further maturation of virus particles in cells infected at low temperature in the case of Q values greater than 1 (see C.III.2.c.).

(c) Temperature Shift during Propagation of Infection

(a') Ni 2519 on Xanthi Tobacco, shift from 23° → 32°C. Fully grown Xanthi plants, cut down to four complete expanded leaves as in the infectivity test, were inoculated with a diluted homogenate of Samsun leaf material infected with Ni 2519, and were then placed in a climate chamber at 23°C under continous illumination. At various times, the plants were placed at 32°C and 24 hours later, they were moved to the green house (20-25°C) for "development" of necrosis. Nine days later, the necrotic lesions were counted.

Figure 1 shows the number of necroses plotted as a function of the time post-infection of the shift from 23°C to 32°C. The number of necroses begins to increase sharply between the 12th and 16th hour post-infection. The point to

the initiation of lesions is still inhibited by high temperature (> 12 hours post-infection) is so late that one must assume that the ts step does not involve the primary processes of infection (penetration of the virus particle, release of the RNA) but rather a later, intracellular, virus-specific function.

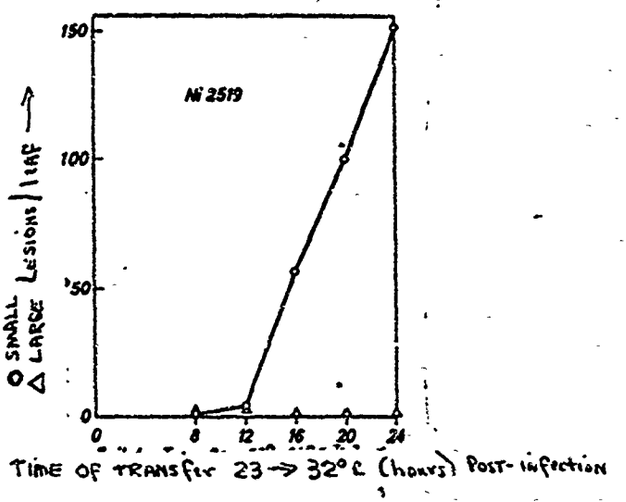

Figure 1. Ni 2519 on Xanthi tobacco. Plot of the number of macroscopic lesions as a function of the time post-infection of the transfer 23°C → 32°C. After incubation at 32°C, the plants were held in a green house at 20-25°C for "development" of the lesions. The large lesions are wild-type contaminations.

(b') Ni 118 and Ni 2519 on Samsun Tobacco, Shift from 32°C → ca. 20°C

Samsun plants were inoculated with 0.1 % suspensions of Ni 118 and Ni 2519 as described in Section C.1. As controls, Xanthi plants were inoculated with the same suspensions. The Samsun plants were smeared with TMV-vulgare antiserum (titer 128, diluted 1/10 with phosphate buffer, pH 7.0). ca. one hour post-infection and were held at 32°C (continous illumination, 95 % relative humidity). After 15 minutes, the antiserum was washed away with water and the zero-time plants were placed at 20-25°C for 8 hours in a green house. Two other groups were placed in the green house for 8 hours at 32°C. after the 12th and 36th hour post-infection. All of the plants were exposed to indirect daylight during the 8 hours in the green house. The control plants were left up to 44 hours post-infection at 32°C. Prior to extraction, leaf material was cooled to 4°C. The

extraction was carried out as described in Section B.IV.1. 1/1 and 1/10 dilutions of the extracts were tested on Xanthi plants.

Shown in Figure 2 are the infectivities that remained. In the case of the short-period post-incubation at low temperature, there developed on the leaf inoculated with Ni 118, a large number of stable viruses at the higher temperature, without production of the virus particles as the controls showed. This indicates, therefore, that the defect of Ni 118 is only a defect in maturation. In the case of Ni 2519, this rapid increase in infectiousness was not observed when the temperature was decreased. According to Section C.III.2.c.a', it is assumed that the infectiousness can generally not be displayed in tissues at high temperatures. The fact that high infectious virus preparations were employed for these studies is shown by the > 1000 lesions per leaf that were observed on the infected control Xanthi plants.

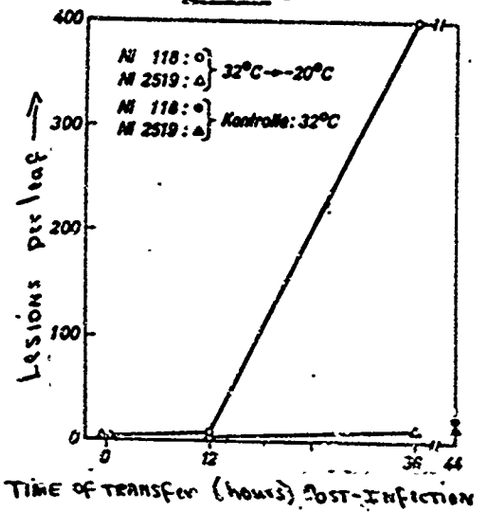

Figure 2. Ni 118 and Ni 2519 on Samsun tobacco. Plot of the number of viruses formed at 20°C after 8 hours against the time of transfer 32° → 20°C. Controls: plants extracted with previously being transferred to 20°C.

IV. Labile Infectivity (RNA)

These studies were designed to show whether or not ts mutants accumulate

RNase-sensitive infectivity at high temperatures. According to the results of Sarkar (1965), the test is most effective when used with a short incubation time.

Six week-old Samsun plants were inoculated as described in Section C.I. with vulgare, Ni 118, Ni 2519 ($\frac{0.1}{.01}$ % virus suspension of all these) and Ni 2204 leaf extract in phosphate buffer, pH 7.

The leaves were washed immediately and treated 30 minutes later with vulgare antiserum on a cotton swab (initial titer: 128, diluted 1:15 with phosphate buffer, pH 7). After an additional 15 minutes, it was washed once more. Two hours later, two plants per each strain were placed at 23°C and 35°C in climate chambers. At the same time, 10 leaf cuttings from inoculated control plants were frozen as zero-time controls. After 50 hours, the plants were extracted as shown in Scheme 1.

SCHEME 1

Extraction of Leaf Material for RNA Detection

ice bath { 10 cuttings, 13 mm diamter from 2 leaves
+ spatula tip of carborundum
+ 2.0 ml buffer "N"

1.0 ml + 4.0 ml buffer "TP" + several mg bentonite	1.0 ml + 4.0 ml phosphate buffer, pH 7.0
↓ 0°C, 2 hr	↓ 20°C, 2 hr
1.0 ml + 1.0 ml phosphate buffer + 3.0 ml "TP"	1.0 ml + 4.0 ml "TP"
↓	↓
Test "T"	against Test "P"

(In contrast to all the rest of the studies, no chloroses were seen on the plants inoculated with Ni 2519 and incubated at the higher temperature.) As test plants, young (8 week old) xanthi plants were employed whose leaves were thinly coated with a 1:1 mixture of bentonite and carborundum (Sarkar, 1965). The "T" and "P" samples for the same incubation temperature were compared by the half-leaf method. Labile infectiousness should be present in the "T" sample, but destroyed in the "P" sample by the leaf RNase (quotient Q= infectiousness of "T"/ infectiousness of "P" >1). Table 4 shows the values found. These are the means of two Q values determined with independent samples from six leaf halves each.

Table 4

Labile Infectiousness (RNA) at 23°C and 35°C

Strain	Zero-time control lecions per half leaf	Incubation temperature	Lesions per half leaf for sample "p"	Q ("T"/"p")
Vulgare	1	23°C	10 $\sim$ 500 $\simeq$ 5000	$\sim$ 0.7
		35°C	10 $\sim$ 700 $\simeq$ 7000	$\sim$ 0.7
Ni 118	6	23°C	$\sim$ 500	$\sim$ 0.7
		35°C	10	1.7 $\pm$ 0.4
Ni 2204		23°C	4	17.7 $\pm$ 2.5
		35°C	5	8.2 $\pm$ 1.0
Ni 2519		23°C	105	0.8 $\pm$ 0.3
		35°C	22	3.2 $\pm$ 1.1

According to Sarkar (1965), this test is approximately 20 times less sensitive for labile RNA than for intact virus when similar particle numbers are compared. His L, the number of lesions remaining, L_t for the "T" buffer extract,

L_p for the phosphate buffer extract is then $L_t = aV + a/20\ N$, and $L_p = aV$ if V and N are the concentrations of virus particles and nucleic acid respectively. a is the "plating efficiency" of the virus. Thus, it follows: $Q = L_t/L_p = 1 + 1/20\ N/V$. If an error of ± 20 % can be assumed for the quotient, then N/V must always be greater than 4 in order to measure a Q of 2. The test will show only a large excess of labile RNA when intact viruses are also present.

Considering this methodological restriction, it can be concluded that (a) Ni 2204 accumulates labile RNA at high and low temperatures, but more at the low temperature. At the same time, this finding confirms the presence of real labile RNA under these test conditions. (b) If the 35°C Q value for Ni 118, which is greater than 1, is actually valid, that is, free RNA is present, then the RNA content corresponding to intact virus is at the very least several times smaller than that of Ni 2204 at 35°C. (c) The same holds true for Ni 2519. However, in this case, the Q value, in contrast to that of Ni 118, is clearly much greater than 2 suggesting that there is probably only a weak accumulation of labile infectiousness at the high temperature. Aside from the question of labile RNA, the studies show that ts and def behavior by the mutants as compared to the behavior of vulgare is also evident after a very short incubation period, a fact that serves as another reason for the small level of infectiousness at ≥ 30°C in the case of these mutants in addition to those given in Section C.III.2.b.

V. Detection of Defective Sheath Proteins in Plants

Based on the hypothesis that the class ts-I mutants produce no stable virus or very reduced quantities at high temperatures because of the conformation of their sheath protein subunits is thermal-labile, it can be predicted that the infected plant cells should contain at high temperature considerable quantities of sheath protein polypeptide which is similar to that of the wild strain vulgare

but in a different conformational state. This prediction has been confirmed with the ts-I mutants Ni 118 and flavum.

With regards to the technical accomplishment, the following report from preliminary experiments was decisive: (a) The defective protein cannot be detected serologically in supernatants obtained by low-speed centrifugation of extracts (pH 7) obtained from infected plants. (b) If pH 8 extracts are obtained using sulfhydryl (SH) protection and then dialyzed to pH 5 after removal of particulate, cellular-derived particles, no rod-shaped aggregates can be observed under the electron microscope. (c) Ni 118 virus sheath protein, which has been denatured at 30°C and precipitated at pH 5 (see Part II), will no longer go into solutionsin aqueous buffers. However, it is easily solubilized in 67 % acetic acid or in 6-10 M urea at pH $\gtrsim$ 7. (d) If one extracts leaf tissue homogenates directly with 10 M urea at pH 9, and dissolves the virus therein, then the viral sheath protein cannot be detected by polyacrylamide gel electrophoresis (see Section B.IV.5) although virus alone in 10 M urea gives a band. Often, alkaline urea solutions extract from the plant a substance to which the virus protein has been adsorbed. This effect is not achieved if one extracts the plant tissues with 67% acetic acid[5] and exchanges this solvent by dialysis of the proteins against urea.

The protein extracted from the tissue must be made insoluble in aqueous buffer by denaturation and detected using a test which characterizes the protein independently from the native state as TMV sheath protein. Solubility in aqueous buffer can be employed as an analytical criterion for the native state.

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[5] The extraction of infected plant tissues with concentrated acetic acid for the detection of insoluble TMV protein was independently carried out by other authors (Parish and Zaitlin, Virology, 30, 1966).

Six-week old Samsun plants, trimmed to four leaves, were inoculated as described in Section C.1. with a 1 % virus suspension of vulgare, Ni 118 and flavum. Control plants were treated in a similar manner with phosphate buffer. After 1.5 hours at room temperature, all the plants were treated with vulgare antiserum (titer 128, diluted 1:10 in phosphate buffer, pH 7) and subsequently placed in a climate chamber at 30°C, 95-100 % relative humidity and 5,000 lux intensity illumination. After five minutes of treatment, the antiserum was washed off with water. Leaves from a vulgare-infected plant which was used as a control for antiserum activity were homogenized in phosphate buffer and the homogenate was tested on Xanthi tobacco. The study plants were incubated for four days in the climate chamber. Because of a deficiency in the climatic controls, the temperature on day 2 dropped to 26°C for 4 hours. This did not, however, influence the conclusions drawn from these studies. The extraction of the leaf materials was carried out according to Scheme 2. 60 gm of leaf cuttings were always put into a final volume of 6 ml of urea solution so that 1 ml of the test solution contained the extract from 10 gm of fresh leaf material. For control purposes, Samsun plants were inoculated with the defective strain PM 2, held for 6 days in the green house at 20-24°C, and then extracted in the same manner.

The following parameters were measured:

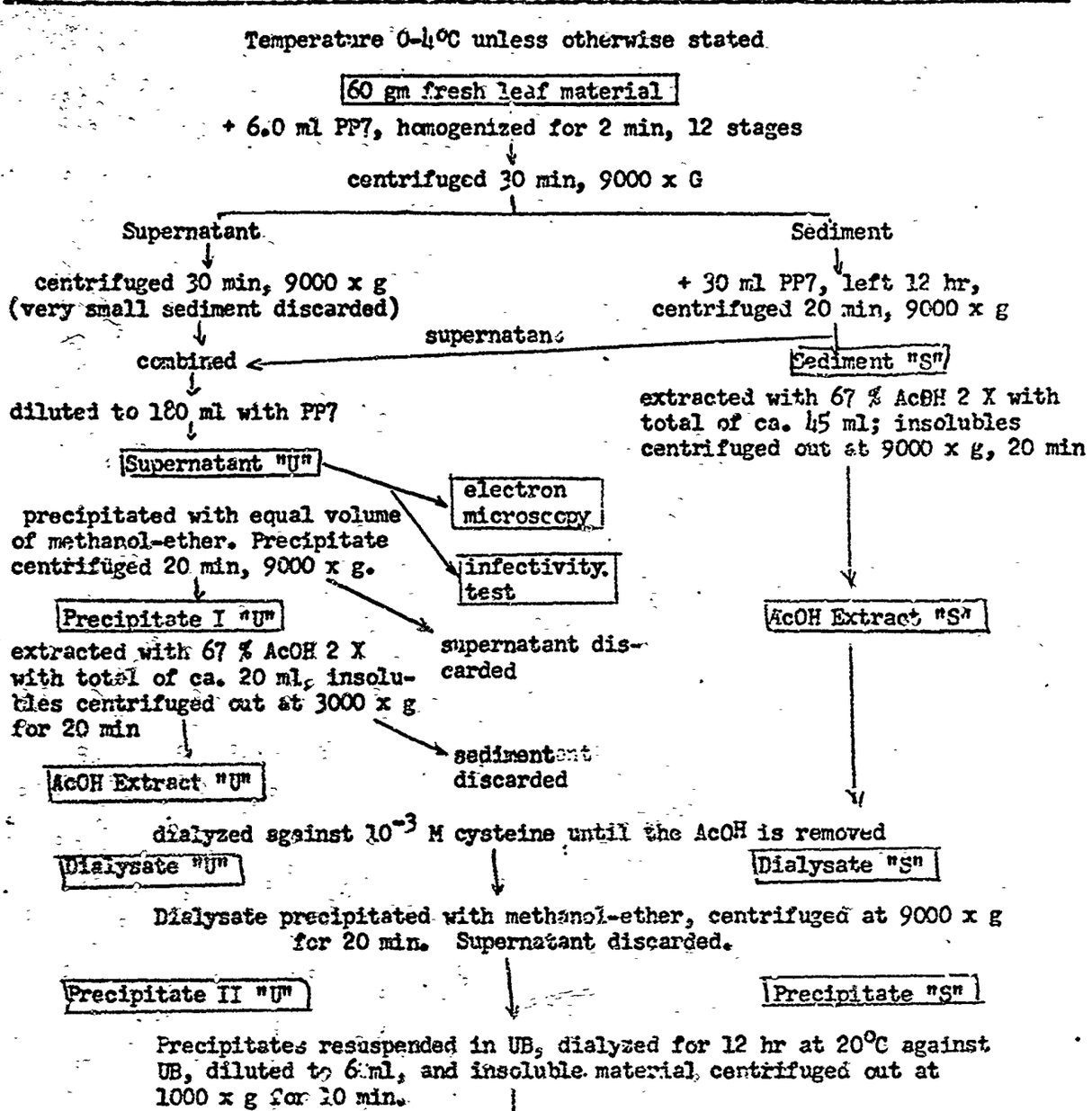
(a) Stable infectivity in phosphate buffer extracts (see B.IV.1.).

(b) Concentration of viral particles in phosphate buffer extracts (see B.IV.3).

(c) Quantity of sheath protein in the supernatant and in the sediment of phosphate buffer extracts (see B.IV.5).

The values that were obtained are summarized in Table 5. Figures 3 and 4 show the results of the polyacrylamide gel electrophoresis experiments.

Scheme 2. Extraction of Leaf Material for The Detection of Defective Sheath Protein



Solutions employed: PP7: M/15 phosphate buffer, pH 7, 10^{-2} M cysteine, 10^{-3} M EDTA. AcOH: 67 % acetic acid, 10^{-2}M cysteine. (Both solutions are supersaturated with cysteine at 4°C). Ethanol-ether: 1:1 mixture, 10^{-3} mercaptoethanol. UB: 8 M urea in Aronsson-Gronvall buffer, pH 8.8, 10^{-3}M cysteine, 20 % sucrose.

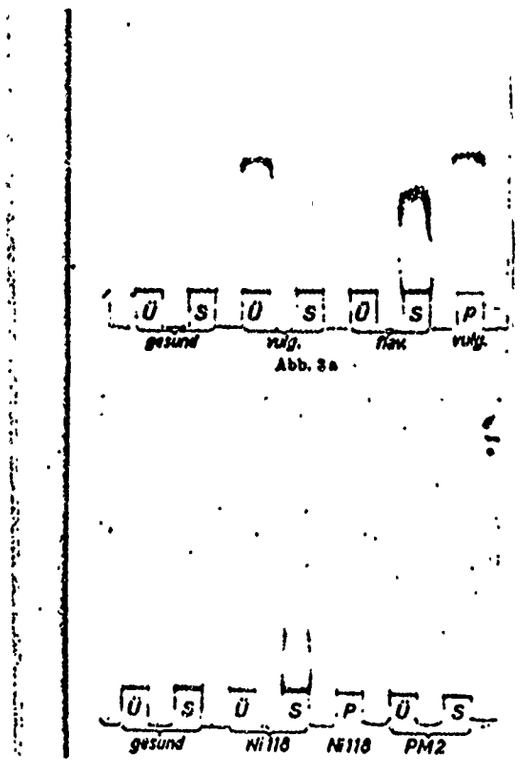


Fig. 3a and b. Electrophoretic patterns of 10-fold concentrated extracts of healthy and TMV-infected tobacco plants incubated at 30°C. 8 M urea, pH 8.8, 200 V, 48 → 30 mA, 15 hour run. U: supernatant, S: sediment of the phosphate buffer extracts. P: protein from viruses, 1 mg/ml. a and b are different runs.

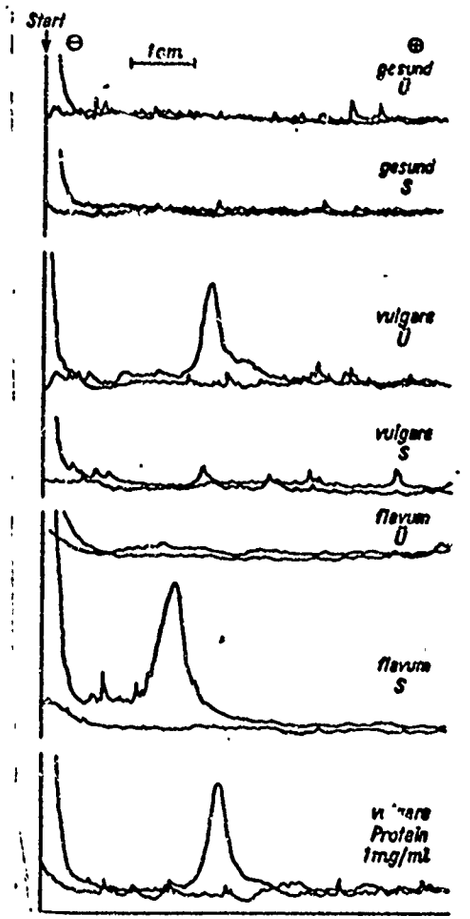


Figure 4. Densitometer curves of gels shown in Figure 3 a. Conditions are those already described.

Table 5. Virus-specific products in the extracts of infected tobacco plants incubated at 30°C

Strain	Infectivity lesions/half-leaf	Relative Infectivity	Virus rods per 100 μ^2	Relative virus rods	Specific Infectivity Lesions/ relative particle	Sheath protein mg/gm leaf tissue
Vulgare	159·250[a] 132·250[b] $\sim 3.6 \cdot 10^4$	1	503·8 $\sim$ 4·103	1	1	U $\sim$ 0.1 S $\sim$ 0.005
Ni 118	7·10 = 70	$2 \cdot 10^{-3}$	1	$2.5 \cdot 10^{-4}$	$\sim$ 10	U 0 S $\sim$ 0.1
Flavum	4·250 = 10^3	$3 \cdot 10^{-2}$	14*	$3.5 \cdot 10^{-3}$	$\sim$ 10	U 0 S $\sim$ 0.1**

* Estimated absolute value: 10^9/ml

** Flavum protein gives a somewhat stronger band than an equal quantity of vulgare protein.

Infectivity in lesions per Xanthi half leaf x dilution factor. Vulgare: [a] parallel to Ni 118, [b] parallel to flavum. Zero-time control for vulgare: 5 lesions per half leaf. Particle count in virus rods per 100 μ^2 field. In the case of vulgare, it was a 2 x 6.25 μ^2 area while for Ni 118 and flavum, it was the 100 μ^2 field. The fields counted contained proportionately a great number of tobacco ribosomes and a few chloroplastids. Specific infectivity in lesions per viral particles as compared to vulgare = 1. Protein in mg/gm fresh leaf tissue was determined by a comparison of areas under the densitometer curves for the extracts with those for purified proteins of the same strain. All values were 0 for the control plants.

Table 6. Amount of visable protein precipitate sedimented after methanol-ether precipitation and centrifugation as described in Scheme 2.

Fraction	vulgare	Ni 118	flavum	PM 2
Precipitate II U	++	0	0	+
Precipitate S	(+)	+	++	0

Text, continued from Page 24

The measurement of these parameters yielded the following information:

(a) The stable infectivity of Ni 118 amounts to ≈ 2 % that of vulgare; the stable infectivity of flavum amounts to ≈ 3 % that of vulgare.

(b) The infectivity found for flavum cannot be attributed to the inoculum since it is ca. 100 times higher than that of the vulgare zero-controls treated with antiserum.

(c) The number of viral particles detectable by electron microscopy in the case of Ni 118 amounted to ∿ 0.02 % that of vulgare and for flavum ∿ 0.3 % that of vulgare.

(d) From a and c, it can be concluded that the specific infectivity measured as lesions per viral particle for Ni 11 8 and flavum at 30°C lies about one log high[illegible] of vulgare.

(e) The total content of sheath protein detectable by electrophoresis was of the same magnitude for vulgare, Ni 118 and flavum, namely, at 0.1 mg per gm of fresh starting material. However, about 95 % of the vulgare protein was found in the supernatant of the phosphate buffer extract while approximately 100 % of the protein of Ni 118 and flavum was found in the sediment. The ts proteins thus exist in a different state in the tissues than the vulgare protein: in the form of rod-shaped aggregates, the vulgare protein is soluble in aqueous

solvents whereas the proteins of both the ts mutants are usually denatured and thereby rendered insoluble.

(f) [illegible] to the findings of Siegel, Zaitlin, and Sehgal (1962) and the control stu[illegible]?, the PM 2 protein is found in the supernatant of aqueous extracts under these conditions. Thus, non-functional TMV sheath protein can exist in the native state (PM 2) or denatured (ts-I mutants at $\geq$ 30°C) in the cytoplasm.

The behavior of various proteins recovered by Scheme 1 and gel electrophoresis was different. In the case of the last methanol-ether precipitation, a distinct difference was apparent in the quantity of precipitated protein between the different species as shown in Table 6. Since the behavior of the first three strains exactly parallels the electrophoretically recovered constituents for viral sheath protein, then the conclusion can be drawn that the recovery according to Scheme 2 results in an overall concentration of viral sheath protein as compared to the plant protein. In contrast to the purification techniques for virus, X protein (Takahashi and Ishii, 1952) and PM 2 protein (Siegel, Zaitlin, and Sehgal, 1962), this is independent of the native state of the proteins. Concerning the absolute yield of this concentration, however, no statements can be made. During gel electrophoresis, vulgare and flavum exhibit well defined bands whose positions are clearly distinquished as expected from the distinct charge capabities of these proteins (Friedrich-Freska et al., 1946) (See Part II). Ni 118 protein (from the plant or virus) exhibited a weak and diffuse band. PM 2 exhibits no band, however behavior during extraction, it can be concluded that the PM 2 protein is located in the supernatant fraction (for behavior in urea, see Part II also).

VI. The Nature of Viral Particles Synthesized at High Temperatures

1. Genetic Properties

The few viral particles formed at high temperature in leaf tissues infected with a ts strain can be (a) selected-out tr revertants or tr contaminants, or (b) may still posses the ts genotype. These possibilities can be differentiated from each other in the following manner: leaf material infected with ts strains is incubated at the high temperature, extracted, and the extract used to inoculate Xanthi tobacco. Random samples of the few lesions which then appear are excised and each isolate is propagated on a Samsun plant at high temperature. The leaf material thus obtained is used for the inoculation of Samsun plants for the temperature test (as in Section C.I.). During the preliminary experiments, it was found that 9 of the 10 Ni 116 lesions obtained from 32°C leaf material and 10 of the 10 flavum lesions still possesed the ts genotype. The possibility could not be excluded, however, that a fraction of the lesions originated from the residues of the first inoculations since in this study, no antiserum was employed. On the basis of Section C.IV., this objection is not practical in the case of flavum lesions which originate from extracts from studies on the detection of sheath proteins. 20 such lesions were excised and tested as described above. During the intermediate propagation on Samsun, 19 lesions demonstrated typical flavum symptoms, whereas one isolate produced a yellowish-green mosaic. For the temperature test, a Xanthi plant (four large leaves) was inoculated per isolate. Two isolates were held as controls at 21-26°C. The number of lesions produced per test plant were as follows: after incubation at 32°C, 15 isolates demonstrated no lesions while 5 isolates had one lesion each. Controls: 439 and 236 lesions. All of the 20 isolates were thus ts. The small level of infectiousness remaining at high temperatures is not the result of tr viruses that are selectively concentrated from the infectious viral particles.

2. Phen[illegible]acteristics

In this regar[illegible] no specific investigations were carried out. Friedrich-Freska et al. (19[illegible] the following observations concerning flavum:

(a) The yield of viral propagation was < 5 % that of vulgare.

(b) The few flavum viruses that were obtained migrated more slowly in an electric field than did vulgare in alkaline solvents. If one assumes that the flavum strain studied in those experiments is identical to that employed in our investigations, as is indicated by the biological criteria and electrophoretic behaviors, then one must conclude that (a) the virus was probably synthesized at moderately high temperatures, and (b) the virus that was synthesized had in its sheath protein the amino acid substitution (aspartic acid → alanine) that leads to temperature sensitivity (Wittmann et al., 1965). In spite of the possibility of temperature variations, one can conclude that at high temperature, neither the genotypic nor the phenotypic deviations from flavum are concentrated (purity of the inoculum and a not-too-long incubation time are assumed.)

VII. Secondary Propagation of ts Virus in The Plant

The secondary propagation of infectivity without the production of large quantities of stable viral particles can be observed best with the yellow strains whose symptoms are very distinct. Mundry (1957) determined that the probability of the expression of secondary symptoms of flavum decrease to $\sim$ 5 % (of the infected plants) at 32°C. This finding has been confirmed. In agreement with the findings of Siegel, Zaitlin, and Sehgal (1962) on the defective mutants PM 1 and PM 2, he explained that stable viral rods are generally necessary for the secondary propagation in the plant but not for the growth of a primary infection focus. The somewhat more stable reflavescens strain (see Part II) undergoes secondary propagation at 32°C, however, the secondarily infected leaves, which are completely whitish-yellow, contain < 1 % of the quantity of

virus which is found in corresponding leaves of vulgare-infected plants. Relatively few stable viral particles are sufficient in this case for the secondary propagation of the infection.

D. SUMMARY OF THE BIOLOGICAL PROPERTIES OF MUTANTS

I. ts Mutants of Class I

The best studied are the mutants flavum and Ni 118. These are characterized by the following properties: at high temperatures ($\geq$ 30°C), none or only small amounts of viral particles are produced. Infectiousness, however, is displayed in the leaf tissue, and a protein is accumulated whose polypeptide chain is identical to that of the sheath protein produced by the viral particle at lower temperatures. The protein does not exist in the native state in the tissues. Large quantities of free RNA are not accumulated. For Ni 118, it has been shown that the infected tissues, immediately after reverse transfer to a lower temperature, produce intact viral particles. The conclusion is that the single temperature-sensitive function of these ts mutants is the maturation of viral particles and that the mutation involved in these cases is located in the sheath protein cistron. Ni 2204: this mutant produces at low temperature in contrast to its parent strain, A 14, strongly reduced quantities of virus. In contrast to the typical ts mutants, large quantities of free RNA are accumulated, at both high and low temperatures. Since it can be shown by means of in vitro studies (Part II) that the defect of this mutant is still associated with the sheath protein, then it still belongs to class I as does flavum, Ni 118, and Siegel's defective mutant PM 2. In class I, therefore, there are mutants which (under restrictive conditions) accumulated large quantities of RNA and those that do not.

II. Ni 2516 and Ni 2519

Both of these mutants are ts, however, according to Wittmann-Liebold et al (1965), no amino acid substitution can be found in their sheath proteins when

they are compared to the sheath protein of the tr parent strain, A 14. In alkaline solvents, Ni 2519 protein migrates in gel electrophoresis about the same speed as does A 14 protein thus excluding the possibility of an amide substitution (Part II). In the in vitro test, Ni 2519 protein is stable at both 30°C and 35°C (see Part II). Ni 2519 shows no or arrested symptom production at high temperature, and at green house temperature, it produces lesions on Xanthi tobacco which are smaller than those of A 14. Since, according to temperare reverse-transfer studies, the defect involves neither a primary event of infection nor the maturation process, then it must involve a temperature-sensitive function necessary to the propagation of infectivity in tissues. The mutation leading to temperature-sensitivity in this case does not lie in the sheath protein cistron but in another cistron of unknown function, which should be designated accordingly as II. Ni 2519 is thus ts-II. Ni-2516 has not yet been sufficiently characterized biologically. It appears, however, to differ substantially from Ni 2519.

III. Ni 2338

This mutant is "inversely temperature sensitive" (its). Perhaps the sheath protein is ts, but only at 35°C. The symptoms and its behavior are dependent on the host employed. In contrast to all the other mutant studied in this report, it causes necrosis primarily on Samsun tobacco. Since the necrotic effect on the plant reduces the virus yield (Samuel, 1957), the its effect can be explained as an indirect host effect and as such, will not be studied further in this work.

E. GENERAL CONCLUSIONS

I. Functions of the TMV Genome

Wittmann (1962) found that in the case of the TMV mutants, which were

produced with nitrous acid, certain amino acids in the sheath protein could be 100 % converted to another. This proved that the structural gene of the sheath protein in its active form depends on viral RNA. According to the triplet code theory, for 158 amino acid residues, there should be ca. 500 nucleotides. Since the total length of the TMV RNA represents ca. 6,500 nucleotides, then one would expect some 10 to 12 additional structural genes for other polypeptide chains whose intracellular functions have been described in TMV replication (Wittmann, 1962). By studying ts mutants of TMV, at least one such function has been detected which in the case of the ts mutant Ni 2519, could be temperature-sensitive (Wittmann-Liebold et al., 1965, and this work) and perhaps another for Ni 2516. The mutants Ni 2519 and Ni 2516 were, however, isolated after employing a special selection procedure. It is still not fully understood why all the other ts mutants belong to Class I, that is, are ts with regards to the sheath protein, when so many other proteins are determined by the TMV RNA. The following possible explanations exist: (a) this effect is a selection artefact in that under the conditions of isolation of the mutants, ts mutants, which cannot be propagated in tissue at high temperature in contrast to the ts-I mutants, are either selectively excluded or through selection of symptomatic divergent strains, certain sheath protein mutants are concentrated. The second hypothesis at least agrees with the yellow strains (see v. Senghusch, 1965). (b) The sheath protein particularly has many amino acid positions which can be mutated to cause temperature-sensitivity. This explains the high proportion of temperature-sensitive mutants which possess an amino acid substitution in the sheath protein (16 out of 21). (c) In the case that both of these explanations are not adequate, then one must assume that the TMV genome contains only 2-4 cistrons which code for protein.

II. The Intracellular State of Defective ts Proteins

Hršel and Brčák (1964), during their investigations into the hypersensitive reaction of Nicotiana glutinosa, incubated flavum-infected leaf cuttings at 30°C and examined the tissues for chloroses elicited at high temperature under the electron microscope. They found fine-structured inclusions in the cytoplasm but no viral rods. This cannot, as the authors concluded, be an effect of plant hypersensitivity since the tr strain itself replicates well in N. glutinosa and also on Xanthi tobacco at high temperature (Kassanis, 1957; Jockusch, 1966a). It is probably for that reasons that Hršel and Brčák were able to observe the defective ts protein under the electron microscope. Their findings were, in any case, in agreement with the data presented in this work in that (a) flavum forms scarcely electron microscopically detectable viral particles at 30°C, and (b) its proteins must exist in the form of insoluble aggregates under these conditions. The relationship between yellow symptoms and ts behavior is no longer surprising since v. Sengbusch could show using Wittmann's mutants that a relationship between yellow symptoms and the primary structure of the protein exists (this correlation can be developed further with knowledge of viral specific enzymes - Friedrich-Freska et al., 1964). The relationship between both phenomena is probably to be found in the solubility properties of the sheath proteins.

III. Reciprocal Stabilization of Protein and RNA in The Cell

1. Possible Stabilizing Effects of Sheath Protein on RNA

During the studies on labile RNA from defective mutants of class I, two behavior patterns were found: (a) RNA was accumulated at 150-300 times more than stable viral particles (Ni 2204). (b) The infectivity was increased, but resulted in practically no viral particles while the RNA was synthesized, if at all, to

about a 10-fold excess over the small quantity of virus (Ni 118 at 35°C). The possibility that in the second case, the TMV RNA was active as messenger RNA was shown by the fact that it could code in the case of vulgare for sheath protein that could be detected by electophoresis. High temperatures cannot be the sole reason for the disappearance of RNA since Ni 2204 accumulates large quantities of RNA under these conditions. The capability to accumulate RNA in large excess in the cell or not, when no viral particles are formed, is thus strain-specific. The defective mutants, PM 1 and PM 2, accumulate it also under green house conditions (Siegel, Zaitlin, and Sehgal, 1962). It is not unlikely that this capability can be dependent on the primary structure of the sheath proteins. In this case, a protein determined by a polycistronic messenger RNA would determine the longevity of the messenger RNA in the cytoplasm.

2. Stabilization of Proteins by the RNA

During the detection of defective sheath proteins of the ts-I mutants, Ni 118 and flavum, it was shown that at 30°C, the specific infectivity of these mutants, measured as lesions per viral particle, was about 10 times higher than that of vulgare. Lebeurier and Hirth (1964) found that in the case of the wild strains employed by them, viral particles were produced at high temperature which had a strongly reduced RNA content. The fact that the wild strain employed here, vulgare, had a smaller specific infectivity in lesions per rod at high temperatures as compared to low temperature indicates that vulgare itself behaves like these strains used by Lebeurier and Hirth. Oehlen (unpublished) found with vulgare that when it was propagated at 32°C, there was a decrease in the UV extinction ratio, E_{260}/E_{280} indicating a diminished RNA content. From these results, the different specific infectivities of ts and tr strains can be explained on the basis that the sheath protein subunits in the presence of RNA

are thermal-stabilized like some enzymes in the presence of their substrates. Vulgare subunits, however, can still form their native quaternary structures without the stabilizing influence of RNA.

IV. Employment of Temperature Sensitivity with TMV

Two possible applications for ts mutants are presented: (a) through temperature variations, certain functions (for example, maturation of ts-I mutants) can be synchronized. This would facilitate kinetic investigations in this system. (b) Assuming that the polypeptide chain is not acetylated by a viral specific enzyme which can itself be made defective by mutation, the capability of ts-I mutants to producing growing lesions on Xanthi at high temperature is a feature that can be easily tested for with large numbers of viral clones in order to detect mutations in the sheath protein structural genes.

SUMMARY

Twenty-one mutants of TMV isolated by other investigators, five specially selected mutants, and four wild strains (Table 2) were investigated with respect to their temperature behavior in the host cell. Of the 21 mutants, 14 were temperature sensitive (ts) in the sense that in tobacco leaves at 32°C, they produced only a fraction of the stable infective material produced at 23°C. Seven of these mutants and all 4 wild strains were temperature resistant (tr), that is, they produced comparable amounts of infective material at both temperatures. The pattern of tr and ts behavior is similar, but not identical, on three other host plants. The 14 unselected ts mutants all showed spreading symptoms at 32°C (ts-I mutants). Eleven of them produced yellow symptoms on tobacco at 23°C as well as at 32°C (Table 3).

From the newly isolated mutants, Ni 2204 is designated as defective (def) because low virus yields are obtained even at 23°C. Ni 2519 is designated as ts-II, because the spreading of symptoms is inhibited at high temperatures (Table 3).

The ts-I mutants, Flavum and Ni 118, the def mutant, Ni 2204, and the ts-II Ni 2519 were studied more intensively. It was found that:

(a) ts behavior is not caused by degradation of complete viral particles (Ni 118, Ni 2519 - Section C.III.2.b.).

(b) The ts mutants are unable to produce rod-like structures (infective or non-infective) at high temperatures (Ni 118 and flavum compared to vulgare, Table 5).

(c) ts-I mutants accumulate the coat protein polypeptide chain, but in an insoluble denatured state (flavum, Ni 118, Figs. 3, 4).

(d) Ni 118 does not accumulate excessive amounts of labile RNA with respect to virus at 35°C, but Ni 2204 does so (Table 4).

(e) The Ni 118-infected plants produces large amounts of stable virus within 8 hours after being transferred from 32°C to low temperature 36 hours post-infection, but the Ni 2519 infected plants does not (Fig. 2).

It is concluded that with Ni 118 and flavum, it is the maturation of the virus particle which is thermo-sensitive as a consequence of a mutation in the coat protein cistron. This conclusion is generalized for all ts-I mutants on the basis of their symptomology and of the chemical analyses (Wittmann and co-workers) and the in vitro behavior (Part II) of their sheath proteins. The state of the defective protein in the cell and the mutual stabilization of RNA and sheath protein subunits are discussed. In Ni 2519, some function is apparently thermo-labile which is necessary for the spreading of the virus infection in host tissue.

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